

Constitutive Activation of the PrfA Regulon Enhances the Potency of Vaccines Based on Live-Attenuated and Killed but Metabolically Active *Listeria monocytogenes* Strains[∇]

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Recombinant vaccines derived from the facultative intracellular bacterium *Listeria monocytogenes* are presently undergoing early-stage clinical evaluation in oncology treatment settings. This effort has been stimulated in part due to preclinical results that illustrate potent activation of innate and adaptive immune effectors by *L. monocytogenes* vaccines, combined with efficacy in rigorous animal models of malignant and infectious disease. Here, we evaluated the immunologic potency of a panel of isogenic vaccine strains that varied only in *prfA*. PrfA is an intracellularly activated transcription factor that induces expression of virulence genes and encoded heterologous antigens (Ags) in appropriately engineered vaccine strains. Mutant strains with PrfA locked into a constitutively active state are known as PrfA* mutants. We assessed the impacts of three PrfA* mutants, G145S, G155S, and Y63C, on the immunologic potencies of live-attenuated and photochemically inactivated nucleotide excision repair mutant (killed but metabolically active [KBMA]) vaccines. While PrfA* substantially increased Ag expression in strains grown in broth culture, Ag expression levels were equivalent in infected macrophage and dendritic cell lines, conditions that more closely parallel those in the immunized host. However, only the *prfA*(G155S) allele conferred significantly enhanced vaccine potency to KBMA vaccines. In the KBMA vaccine background, we show that PrfA*(G155S) enhanced functional cellular immunity following an intravenous or intramuscular prime-boost immunization regimen. These results form the basis of a rationale for including the *prfA*(G155S) allele in future live-attenuated or KBMA *L. monocytogenes* vaccines advanced to the clinical setting.

Recognition of the advantages of recombinant *Listeria monocytogenes*-based vaccines compared to those of other recombinant-vaccine platforms has facilitated the ongoing development and current evaluation of the former in early-phase clinical trials. These advantages include practical considerations, such as straightforward fermentation methods for manufacturing, and other desirable features, such as the ability to repeat administer even in the presence of protective *L. monocytogenes*-specific immunity (6, 40, 41). One compelling rationale for this vaccine platform is based on the well-known correlates of protection in the mouse listeriosis model: long-lived functional CD4⁺ and CD8⁺ memory T cells induced in response to a single immunization with *L. monocytogenes* (19, 28). There are now numerous publications that demonstrate the striking efficacy of recombinant *L. monocytogenes* vaccines in several animal models due to robust innate and adaptive cellular immunity (9, 10, 29). Recombinant *L. monocytogenes*-based vaccines represent an emerging approach to addressing an acute global need for effective vaccines that elicit functional cellular immunity to prevent or treat infections such as human

immunodeficiency virus infection, hepatitis C virus infection, tuberculosis, and malaria as well as cancer.

As *L. monocytogenes* is a food-borne pathogen having increased virulence among immunocompromised individuals, attenuated vaccine platforms are a prerequisite for advancement to evaluation with humans (23). We have previously described both live-attenuated and photochemically inactivated vaccine platforms derived from the wild-type (WT) strain 10403S (8, 9). The live-attenuated vaccine strain is deleted of both the *actA* and the *inlB* virulence genes (*L. monocytogenes* Δ *actA* Δ *inlB* vaccine strain), which in combination limit growth in the liver, a principal target organ of infection by the WT organism. Liver toxicity in mice, as measured by serum liver function tests for alanine transaminase and aspartate transaminase, is dramatically lower in mice injected intravenously (i.v.) with the *L. monocytogenes* Δ *actA* Δ *inlB* strain than in those injected i.v. with WT *L. monocytogenes*. Furthermore, liver toxicity was minimal and not dose limiting in two toxicology studies performed under good laboratory practice guidelines with cynomolgus monkeys given escalating doses of *L. monocytogenes* Δ *actA* Δ *inlB*-based strains (unpublished data). The *L. monocytogenes* Δ *actA* Δ *inlB* vaccine strain forms the basis for two ongoing FDA-approved phase 1 clinical trials being conducted with adult subjects with advanced cancers. The second vaccine platform, termed “killed but metabolically active” (KBMA), is derived from the *L. monocytogenes* Δ *actA* Δ *inlB* vaccine strain and is deleted of both *uvrA* and *uvrB*, genes encoding the DNA

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repair enzymes of the nucleotide excision repair pathway. KBMA vaccines (*L. monocytogenes* $\Delta actA$ $\Delta inlB$ $\Delta uvrAB$ vaccine strains) are exquisitely sensitive to photochemical inactivation by combined treatment with the synthetic psoralen S-59 and long-wave UV light. While killed, KBMA *L. monocytogenes* vaccines can transiently express their gene products, allowing them to escape the phagolysosome and induce functional cellular immunity and protection against WT *L. monocytogenes* and vaccinia virus challenge (8).

While studies of *L. monocytogenes*-based vaccines have to date been encouraging, all-too-frequently promising preclinical results do not necessarily forecast clinical success. There is a continued need for refinements that either enhance the potency or reduce the toxicity of *L. monocytogenes*-based vaccines in order to facilitate their ultimate clinical development. Here, we evaluated the impact of constitutively active PrfA on the vaccine potencies of both live-attenuated and KBMA vaccines. PrfA is a transcription factor activated intracellularly that acts as a central virulence regulator, serving to enable what has been described as the “Dr. Jekyll and Mr. Hyde” dichotomy of *L. monocytogenes* growth lifestyles in mammals or as a saprophyte (16). PrfA knockout strains are avirulent (43). In WT *L. monocytogenes*, PrfA is expressed upon infection of host cells and, in turn, induces expression of the *prfA* regulon including the *hly* and *plcA* genes, encoding listeriolysin O (LLO) and phospholipase C, respectively. In combination, these gene products mediate escape of the bacterium from the harsh microenvironment of the phagolysosome. PrfA-dependent promoters can be utilized to drive antigen (Ag) expression in recombinant *L. monocytogenes* vaccines by linking the heterologous gene to either the *hly* or the *actA* promoters (references 17 and 36 and this study). Amino acid substitutions in PrfA that result in the constitutive activation of PrfA-dependent genes are known collectively as PrfA* mutants (34, 35). A number of WT *L. monocytogenes* strains with a hyperhemolytic phenotype have a mutation in *prfA*, most commonly G145S, which can result in increased virulence compared to that of laboratory-adapted strains, such as 10403S (34). Similarly, other *prfA* mutants with increased expression of PrfA-dependent genes that were selected by a chemical mutagenesis approach also had increased virulence in mice (38). We hypothesized that induction of PrfA-dependent genes prior to immunization would enhance the efficiency of vaccines through diverse mechanisms, including increase of phagolysosomal escape and expression of PrfA-dependent encoded Ags in the cytosol of the host cell, leading to more-potent CD4⁺ and CD8⁺ T-cell responses.

In this study, we assessed the impacts of three *prfA* mutations on the potencies of isogenic live-attenuated and KBMA vaccine strains. While Ag expression levels were increased in PrfA* vaccine strains grown in broth culture, no differences in Ag expression level between PrfA* vaccine strains and vaccine strains with native *prfA* could be detected in the cytosol from infected macrophage or dendritic cell (DC) lines. Strikingly, however, the magnitude and functionality of vaccine-induced CD8⁺ T cells, as measured by protection against bacterial or viral challenge, were significantly improved among recombinant KBMA *L. monocytogenes* vaccine strains with PrfA*(G155S) compared to those of vaccines with all other *prfA* alleles tested. These findings indicate that activation of

the *prfA* regulon and induction of Ag expression prior to immunization enhance the potency of *L. monocytogenes*-based vaccines.

MATERIALS AND METHODS

Mice. Six- to 12-week-old female C57BL/6 and BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). Studies were performed under animal protocols approved by the Anza Institutional Animal Care and Use Committee.

Bacterial strains. *L. monocytogenes* vaccine strains were constructed in the *L. monocytogenes* $\Delta actA$ $\Delta inlB$ $\Delta uvrAB$ strain (8). PrfA* variants were constructed by cloning the various *prfA* alleles with 800 bp to 1 kb of flanking homology into the temperature-sensitive allelic exchange vector pKSV7 and used to replace the WT allele by using standard procedures (11). Strains with activated PrfA phenotypes were screened for increased zones of both phospholipase activity on egg yolk overlay plates (47) and hemolysis on horse blood agar (Remel). Phenotypically correct clones were confirmed by sequencing the genomic *prfA* locus. An Ag expression cassette termed the Quadvac construct, expressing four vaccinia CD8⁺ T-cell epitopes of various levels of strength and the ovalbumin (OVA)-derived CD8⁺ T-cell epitope SIINFEKL from a single synthetic gene, was designed in silico where the epitopes were strung together and spaced with a linker sequence. The amino acid sequence was codon optimized for expression in *L. monocytogenes* by using Gene Designer software (45), synthesized (DNA2.0; Menlo Park, CA), and cloned downstream of the *actA* promoter and in-frame with the amino terminus of the *actA* gene. The construct was cloned into a derivative of the pPL2 integration vector and stably integrated at the tRNA^{Arg} locus of the bacterial chromosome in the various *prfA** strain backgrounds, as described previously (21). All molecular constructs were confirmed by DNA sequencing.

Western blot detection of heterologous Ag expression. Western blot analyses of broth culture were performed on equivalent amounts of trichloroacetic acid-precipitated supernatants of bacterial cultures grown in yeast extract medium to an optical density at 600 nm of 0.7 (late log). For Western blots of *L. monocytogenes*-infected host cells, J774 cells or DC2.4 cells were inoculated at a multiplicity of infection of 50 or 100 for 1 h, and the cells were washed three times with phosphate-buffered saline and Dulbecco's modified Eagle's medium supplemented with 50 μ g/ml gentamicin. For early time points, DC2.4 cells were harvested at 1.5 or 2.5 h postinfection. For late time points, J774 cells were harvested at 7 h. Cells were lysed with sodium dodecyl sulfate sample buffer, collected, and run on 4 to 12% polyacrylamide gels and transferred to nitrocellulose membranes for Western blot analysis. All Western blots utilized a polyclonal antibody raised against the mature N terminus of the ActA protein.

Immunizations. Live-attenuated bacteria were prepared for immunization from overnight cultures grown in yeast extract medium. KBMA *L. monocytogenes* strains were S59-psoralen and UVA treated as previously described (8). Photochemically inactivated bacteria (KBMA *L. monocytogenes*) were washed once with Dulbecco's phosphate-buffered saline, resuspended in 8% dimethyl sulfoxide, and then stored at -80°C . Bacteria were diluted in Hanks' balanced salt solution (HBSS) for injection. Injection stocks of live-attenuated bacteria were plated to confirm CFU counts. Totals of 5×10^6 CFU live-attenuated bacteria and 1×10^8 particles of KBMA bacteria were administered either i.v. into the tail vein at a 200- μ l volume or intramuscularly (i.m.) into a single tibialis cranialis muscle at a 30 μ l volume, with a boost vaccination given in an alternate tibialis cranialis muscle.

Detection of serum cytokines and chemokines. Serum was collected from mice by retro-orbital bleed at 2, 4, and 8 h postvaccination with KBMA or 4, 8, and 24 h with live-attenuated *L. monocytogenes*. Cytokines/chemokines were measured using a mouse inflammation Cytometric Bead Array kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Samples were acquired using a FACSCanto flow cytometer (BD Biosciences). Data were analyzed using Cytometric Bead Array software (BD Biosciences).

Peptides. The OVA₂₅₇₋₂₆₄ (SIINFEKL), LLO₁₉₀₋₂₀₁ (NEKYAQAYPNVS), HSV-gB2 (SSIEFARL), B8R₂₀₋₂₇ (TSYKFESV), K3L₆₋₁₅ (YSLPNAGDVI), C4L₁₂₅₋₁₃₂ (LNFRFENV), and A42R₈₈₋₉₆ (YAPVSPIVI) (24) peptides were synthesized by Synthetic Biomolecules (San Diego, CA).

Reagents for flow cytometry. CD3 fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-Cy7 (clone 145-2C11), CD4 FITC (clone GK1.5), CD8 PE-Cy7 or Ag-presenting cell (APC)-Cy7 (clone 53-6.7), CD19 FITC (clone MB19-1), tumor necrosis factor (TNF) PE (clone MP6-XT22), and gamma interferon (IFN- γ) APC (clone XMG1.2) were purchased from eBioscience

(San Diego, CA). CD8 α PerCP (clone 53-6.7) was purchased from BD Biosciences (San Jose, CA).

In vivo cytotoxicity assay. Splenocytes from naïve recipients were pulsed with a 1 μ M concentration of either control (HSV-gB2) or target (B8R or A42R) peptide. Cells were then labeled with concentrations of carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) of 0.2 μ M (CFSE^{low}), 1 μ M (CFSE^{med}), or 5 μ M (CFSE^{high}). A total of 3×10^6 labeled spleen cells of each population were mixed and injected i.v. Spleens were harvested 16 h later, and the proportion of target to control populations was determined and the percentage of killing calculated.

Intracellular staining of Ag-specific T cells. Splenocytes were stimulated for 5 h with the relevant peptide in the presence of brefeldin A for intracellular cytokine staining, as previously described (9). Stimulated cells were surface stained for CD4 and CD8 and then fixed and permeabilized using a cytofix/cytoperm kit (BD Biosciences, San Jose, CA). Cells were then stained for IFN- γ , TNF- α , and/or interleukin 2 (IL-2). Samples were acquired using a FACSCanto flow cytometer (BD Biosciences). Data were gated to include exclusively CD4⁺ or CD8⁺ events, and then the percentage of these cells expressing IFN- γ was determined. Data were analyzed using FlowJo software (Treestar, Ashland, OR).

***L. monocytogenes* protection studies.** To assess protective immunity, BALB/c mice were vaccinated with the indicated strains and challenged at 14 days post-vaccination with a 2 \times 50% lethal dose (LD₅₀) of WT *L. monocytogenes* (1×10^5 CFU), and CFU counts in the spleen in organ homogenates were measured 3 days later, as described previously (4). Median lethality (LD₅₀) values were determined as described previously (9).

Vaccinia virus protection studies. C57BL/6 mice were given prime and boost vaccinations separated by 27 days with *L. monocytogenes* Quadvac strains, and 28 days later, mice were challenged intraperitoneally with 1×10^7 PFU of vaccinia virus. Protection was evaluated by measuring viral titer in the ovaries 5 days after virus challenge, as described previously (1).

Statistical analysis. Differences in protection against vaccinia virus or *L. monocytogenes* challenges were determined by Student's *t* test. Unless otherwise indicated, all experiments were conducted at least twice.

RESULTS

Construction and characterization of isogenic PrfA* *L. monocytogenes* vaccine strains. We hypothesized that induction of the PrfA regulon prior to immunization would increase the immunologic potencies of recombinant live-attenuated and KBMA *L. monocytogenes* vaccines. To test this hypothesis, we constructed a panel of isogenic strains on an *L. monocytogenes* background that contained complete coding region deletions of *actA*, *inlB*, *uvrA*, and *uvrB* (*L. monocytogenes* Δ *actA* Δ *inlB* Δ *uvrAB* strains) that varied only in *prfA*. We selected three *prfA* mutants that were generated by chemical mutagenesis or were spontaneous mutants and encoded a constitutively active PrfA* protein (34, 38). Strains with PrfA*(G155S), PrfA*(G145S), or PrfA*(Y63C) have previously been shown to either increase the expression level of an *actA* promoter-dependent β -glucuronidase reporter protein relative to those of isogenic strains with the native *prfA* gene or, in some cases, increase the virulence of WT *L. monocytogenes* (25, 34, 38; M. D. Miner, G. C. Port, and N. E. Freitag, submitted for publication).

To enable us to distinguish immunologic potency differences between isogenic *L. monocytogenes* vaccine strains, we constructed an Ag expression cassette that encoded five well-defined H-2^b-restricted major histocompatibility complex (MHC) class I epitopes that have previously been shown to elicit a range of CD8⁺ T-cell responses in mice. A construct encoding four tandemly spaced vaccinia virus (A42R, C4L, K3L, and B8R) epitopes and the chicken OVA (SL8) epitope was synthesized and then cloned under the control of the PrfA-regulated *actA* promoter as a fusion protein with the 100 N-terminal amino acids of ActA. The construct known as Quadvac was cloned into a derivative of pPL2 and then inte-

grated at the tRNA^{Arg} locus in four isogenic strains (*L. monocytogenes* Δ *actA* Δ *inlB* Δ *uvrAB* strains), each harboring the WT, G145S, G155S, or Y63C *prfA* allele, as described previously (Fig. 1A) (21). The four isogenic vaccine strains all grew equivalently in yeast extract or brain heart infusion broth culture (data not shown). Vaccine strains grown in yeast extract broth culture were either used directly as a live-attenuated *L. monocytogenes* vaccine (9) or photochemically inactivated with the synthetic psoralen S-59 and long-wave UV light to yield a KBMA *L. monocytogenes* vaccine, as described previously (8).

We compared the levels of Ag expression and secretion in broth culture from the four live-attenuated *L. monocytogenes* vaccines, and as expected, higher expression levels were observed in PrfA* strains than in the strain with a WT *prfA* allele (Fig. 1B). While overexpression of PrfA-dependent genes in PrfA* strains grown in broth culture, combined with enhanced invasion of epithelial cells, has been well described (25, 31, 38, 44), little is known regarding whether the PrfA* phenotype is recapitulated in infected, cultured mammalian cells. We evaluated Ag expression from the *L. monocytogenes* vaccine strains in the phagocytic mouse macrophage and DC lines J774 and DC2.4, respectively, rather than in nonphagocytic cell lines, such as HepG2 (liver) or PtK2 (epithelial). The *L. monocytogenes* Δ *actA* Δ *inlB* strain cannot mediate InlB-dependent infection of liver cells expressing the hepatocyte growth factor receptor, and epithelial tissues do not represent a major target that is relevant to the i.m. and i.v. immunization routes used in this investigation. Surprisingly, Ag expression levels in J774 macrophages infected with the isogenic *L. monocytogenes* vaccine strains were equivalent regardless of *prfA* allele (Fig. 1C). Notably, Ag expression levels were also equivalent at early time points in DC2.4 DCs (Fig. 1D). Infection (uptake) and intracellular growth of all *L. monocytogenes* vaccine strains in J774 (Fig. 1E) and DC2.4 (not shown) cell lines were equivalent and not dependent on *prfA* allele.

PrfA* minimally increases the virulence of *L. monocytogenes* Δ *actA* Δ *inlB* Δ *uvrAB* strains. It has previously been reported that PrfA* mutants have virulence levels increased up to 10-fold in BALB/c mice. For example, *prfA*(G155S) decreased the LD₅₀ value of its isogenic WT strain from 2×10^4 CFU to 3×10^5 CFU (38). However, the impact of PrfA* mutants on the virulence of attenuated strains is unknown. As the immunization dose used for *L. monocytogenes* vaccine studies in mice is typically 1/10 the LD₅₀ value, we measured the impacts that the three PrfA* mutants used in this investigation had on the virulence of the *L. monocytogenes* Δ *actA* Δ *inlB* Δ *uvrAB* strain, a strain that is rapidly cleared from the liver following i.v. administration and is attenuated in mice by more than 3 logs compared to the level for WT *L. monocytogenes* (5, 8, 9). PrfA* marginally increased the virulence of the *L. monocytogenes* Δ *actA* Δ *inlB* Δ *uvrAB* strain, with the LD₅₀ value decreased only 2.1-fold (3.5×10^7 CFU versus 7.3×10^7 CFU) in isogenic strains harboring the *prfA*(G145S) or *prfA*(Y63C) allele and 1.4-fold (5.2×10^7 CFU versus 7.3×10^7 CFU) in the isogenic strain harboring the *prfA*(G155S) allele (Table 1).

L. monocytogenes-based vaccines, including attenuated *L. monocytogenes* Δ *actA* Δ *inlB*-based strains, are potent activators of innate immunity, as reflected by the Th1-polarizing, proinflammatory serum cytokine profile induced in response to i.v. administration (4). As activation of innate immunity is related

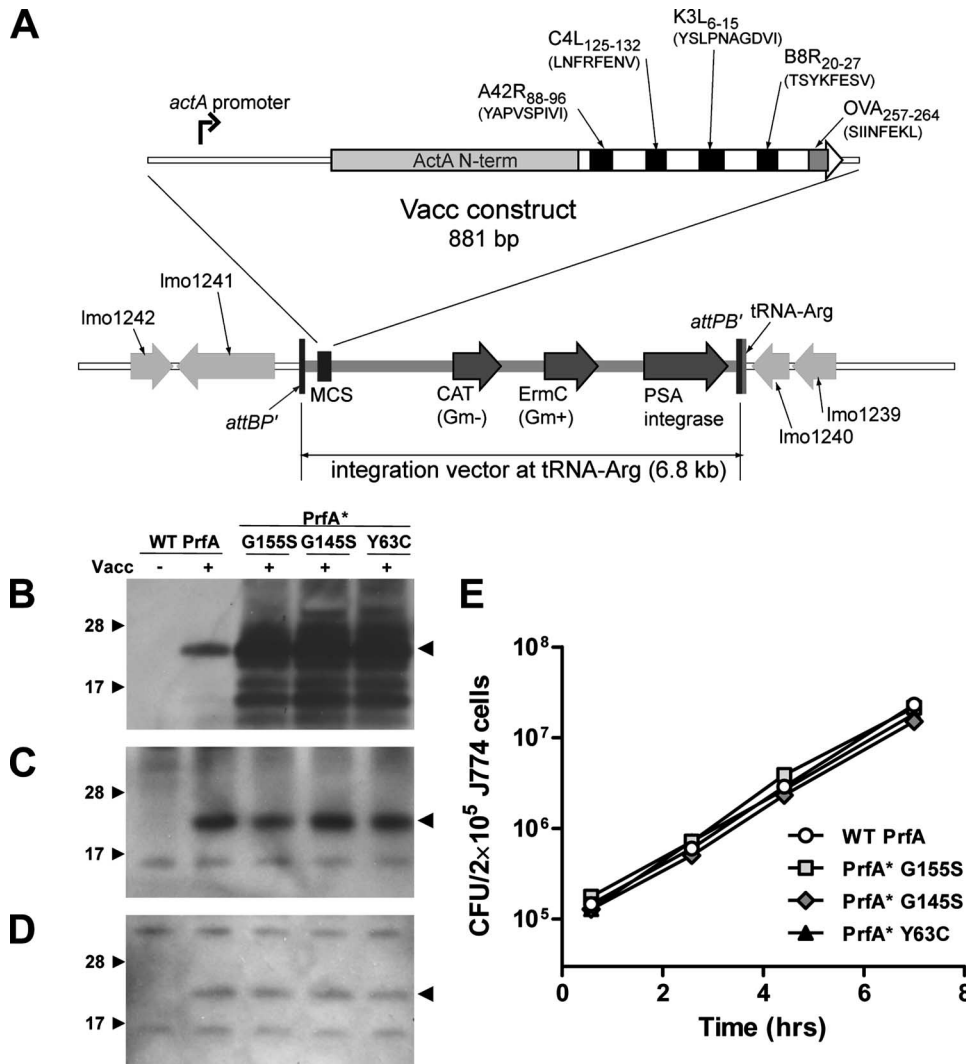


FIG. 1. Characterization of *L. monocytogenes* PrfA* vaccine strains. (A) Construction of the *L. monocytogenes* Quadvac strain expressing four vaccinia virus T-cell epitopes (A24R, C4L, K3L, and B8R) and the OVA SL8 epitope spaced with linker sequences and fused to the first 100 amino acids of ActA (ActAN100) by use of a derivative of the pPL2 site-specific integration vector. CAT, chloramphenicol acetyltransferase; Gm⁻, gram negative; Gm⁺, gram positive. (B) Expression of the heterologous protein in yeast extract broth. (C) Expression of the heterologous Ag at 7 h postinfection in infected J774 macrophage cells. (D) Expression of the heterologous Ag at 2.5 h postinfection in infected DC2.4 DCs. (E) Intracellular growth of isogenic *L. monocytogenes* vaccine strains in J774 macrophages.

to the quality of the *L. monocytogenes* vaccine-induced immune response, we measured the serum cytokine levels at several time points during the first 24 h following i.v. administration of the isogenic vaccine strains. C57BL/6 mice were injected i.v.

with 5×10^6 CFU, a dose that approximated the 0.1-LD₅₀ value for the four isogenic vaccine strains. All three PrfA* vaccine strains induced statistically significantly higher levels of the proinflammatory cytokines/chemokines, such as IL-6 and MCP-1, within 8 hours of administration compared to the vaccine strain with native *prfA* (Fig. 2A). Additionally, PrfA*(G155S) and PrfA*(G145S) induced significantly more TNF- α and IFN- γ . No significant differences between the three PrfA* strains were observed; however, increased mouse-to-mouse variability was observed in mice given the PrfA*(Y63C) vaccine strain.

PrfA* increases the immunogenicity of live-attenuated *L. monocytogenes* vaccines. We evaluated the immunogenicity of the isogenic vaccine strains to assess the impact of PrfA* on vaccine potency. To facilitate comparison, isogenic vaccine strains expressed a common heterologous Ag (termed Quadvac) composed of multiple defined, H-2^b-restricted MHC class

TABLE 1. Virulence of live-attenuated *L. monocytogenes* strains

Strain	Genotype	<i>prfA</i> mutation	LD ₅₀	
			No. of CFU	Fold change relative to WT level
BH1299	$\Delta actA \Delta inlB \Delta uvrAB$	None (WT)	7.3×10^7	
BH1371	$\Delta actA \Delta inlB \Delta uvrAB$ <i>prfA</i> (G155S)	G155S	5.2×10^7	1.4
BH1375	$\Delta actA \Delta inlB \Delta uvrAB$ <i>prfA</i> (G145S)	G145S	3.5×10^7	2.1
BH1379	$\Delta actA \Delta inlB \Delta uvrAB$ <i>prfA</i> (Y63C)	Y63C	3.5×10^7	2.1

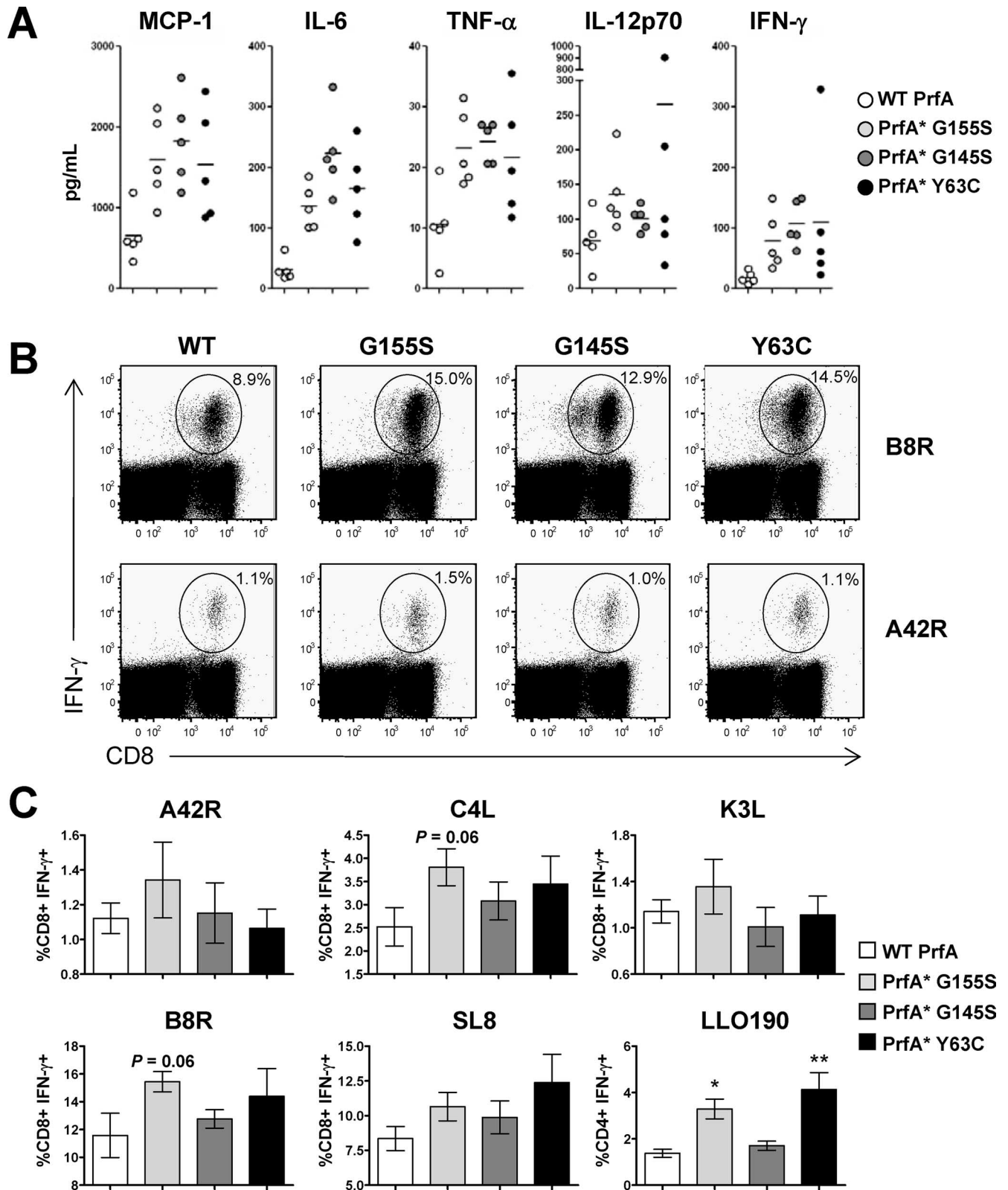


FIG. 2. Improved innate and adaptive immunity induced by PrfA* vaccine strains. (A) Serum cytokine/chemokine levels determined 8 h following a single i.v. administration of 5×10^6 CFU of *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA*, PrfA*(G155S), PrfA*(G145S), and PrfA*(Y63C) strains. Cytokine/chemokine levels were determined by a cytometric bead array. Each symbol represents a single animal. Data are from a single experiment, representative of at least two experiments. The levels of monocyte chemoattractant protein 1 (MCP-1), IL-6, TNF- α , and IFN- γ induced by the PrfA*(G155S) and the PrfA*(G145S) strains were significantly increased over the WT levels ($P < 0.05$); PrfA*(Y63C) was significantly increased for MCP-1 ($P < 0.05$) and IL-6 ($P < 0.005$). (B, C) Live-attenuated PrfA* vaccine strains induced Ag-specific immunity of a higher magnitude. C57BL/6 mice were immunized i.v. with 5×10^6 CFU of *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA*, PrfA*(G155S), PrfA*(G145S), and PrfA*(Y63C) strains. Ag-specific T-cell responses were determined by intracellular cytokine staining at the peak of the response 7 days following vaccination. (B) Dot blots from a representative animal from each group are shown. (C) The means \pm standard deviations are shown for each group of five animals. *, $P < 0.05$; **, $P < 0.005$ for comparison to WT *prfA*.

I vaccinia virus epitopes (A42R, C4L, K3L, and B8R) that have been shown to elicit high-, intermediate-, and low-frequency T-cell responses following vaccinia virus infection and, in addition, the strong OVA epitope SL8. This strategy allowed us both to rank the magnitudes of vaccine-induced CD8⁺ T-cell responses over a dose range of immunization and to evaluate the quality of the response by challenge with vaccinia virus.

Groups of female C57BL/6 mice were immunized i.v. with 5×10^6 CFU of the four isogenic *L. monocytogenes* Quadvac strains, and the CD8⁺ and (*L. monocytogenes*-specific) CD4⁺ T-cell frequencies were determined by intracellular cytokine staining at the peak of the response (9), 7 days following a single immunization. The PrfA*(G155S) *L. monocytogenes* vaccine induced Ag-specific T-cell responses of greater magnitude than the G145S and Y63C PrfA* vaccine strains and the strain with WT *prfA* (Fig. 2B and C). The increased magnitude of the Ag-specific IFN- γ -positive T cells in PrfA*(G155S) *L. monocytogenes* vaccine-immunized mice was greater not only with the immunodominant SL8 and B8R epitopes but, importantly, also with the intermediate- and low-frequency epitopes, A42R, C4L, and K3L. The LLO-specific CD4⁺ T-cell response was increased twofold in mice immunized with the PrfA*(G155S) vaccine strain compared to the level for the other vaccine strains.

PrfA*(G155S) increases the immunogenicity of KBMA *L. monocytogenes* vaccines. We hypothesized that constitutive activation of PrfA and induction of the PrfA regulon might improve the immunogenicity of KBMA *L. monocytogenes* through a variety of mechanisms, including increased escape from the vacuole as well as an increased expression level of the heterologous Ag in the cytosol of APCs. We demonstrated previously that CD8⁺ T-cell potency and protective immunity require that the immunizing *L. monocytogenes* strain access the cytosol (4). As KBMA vaccine strains are unable to expand in the cytosol, increased efficiency of escape from the phagosome through increased expression of LLO and phospholipase C might enhance vaccine potency.

To assess the innate as well as adaptive immunity to KBMA *L. monocytogenes* strains, C57BL/6 mice were immunized i.v. with 1×10^8 particles, a well-tolerated dose. As described previously, we used photochemical inactivation conditions that resulted in 10-log killing of *L. monocytogenes* vaccine preparations (8). Thus, individual mice had a 10^{-2} chance of receiving a single live *L. monocytogenes* $\Delta actA \Delta inlB \Delta uvrAB$ bacterium, a nonimmunizing dose. Serum cytokine/chemokine levels were measured during the first 8 h of infection, which we had observed in previous experiments to include the peak of the response, which then returned to background levels within 24 h (5). KBMA PrfA* vaccine strains induced higher levels of MCP-1, IL-12p70, and IFN- γ than the KBMA vaccine with WT *prfA*; the differences were significant for some of the cytokines (Fig. 3A). No significant differences between the three PrfA* strains were observed, but the levels of cytokines induced by the KBMA PrfA*(G155S) vaccine tended to be higher than the levels induced by the KBMA PrfA*(G145S) or PrfA*(Y63C) vaccine.

We compared the immunogenicities of the isogenic KBMA vaccine strains in C57BL/6 mice, each given two vaccinations separated by 2 weeks. The highest magnitude of the secondary Ag-specific CD8⁺ T-cell response specific for the five Quadvac

epitopes was observed in mice immunized with KBMA PrfA*(G155S), and the four vaccinia epitopes were significantly higher than those for WT *prfA* (Fig. 3B and C). While the magnitude of the CD8⁺ response was generally higher in mice immunized with the other two KBMA PrfA* vaccine strains than in those immunized with KBMA WT *prfA*, this was not the case with all CD8 T-cell epitopes evaluated, with only one epitope from each strain reaching statistical significance (Fig. 3B and C). Interestingly, in contrast to what was found for the live-attenuated vaccine strains, LLO-specific CD4⁺ T-cell responses were not higher in magnitude among mice immunized with KBMA *L. monocytogenes* PrfA* strains. Thus, the *prfA*(G155S) allele conferred the highest immunogenicities to both live-attenuated and KBMA vaccine strains.

KBMA PrfA* vaccines have increased immune potency. It is well established that the magnitude of an induced T-cell response is not necessarily representative of the potency of the response. To assess the potency of the vaccine-induced CD8⁺ T-cell response, we assessed the in vivo cytolytic activities specific for two vaccinia epitopes, A42R and B8R. C57BL/6 mice were immunized twice with the four isogenic KBMA *L. monocytogenes* strains. We evaluated immunogenicity, following two alternative immunization routes: i.v. and i.m. We evaluated i.m. immunization to assess the potency of KBMA PrfA* vaccine strains administered by a conventional vaccination route. Mice were immunized i.v. or i.m. twice, 2 weeks apart, with KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA*, PrfA*(G155S), PrfA*(G145S), and PrfA*(Y63C) strains or vehicle (HBSS) as a control. In vivo cytolytic activity was determined 7 days later by challenging mice with gB2 (negative control; middle peak), A42R-loaded splenocytes (right peak), or B8R-loaded splenocytes (left peak). Robust responses specific for the strong B8R epitope were observed in all groups immunized with KBMA PrfA* or WT *prfA* vaccines. The extent of target killing was slightly higher in mice that were immunized i.v. (Fig. 4A and B). Potent killing activity was also elicited against the A42R vaccinia virus epitope in mice immunized i.v. or i.m. with KBMA PrfA*(G155S). However, the killing activity against A42R induced by KBMA vaccines based on WT PrfA* and given i.m. was reduced twofold compared to that for PrfA*(G155S). KBMA vaccines based on G145S or Y63C were of intermediate potency when given by the i.m. route (Fig. 4B).

KBMA PrfA*(G155S) vaccines elicit protective immunity. While all three of the mutant *prfA* alleles tested conferred increased immune potency to KBMA vaccines compared to native *prfA*, the highest magnitude and degree of in vivo killing specific for A42R, which was the weakest epitope evaluated, was observed with KBMA PrfA*(G155S) vaccines (Fig. 4B). To better characterize the functionality of the CD8⁺ T-cell immunity elicited by KBMA PrfA*(G155S) vaccines, we determined its ability to establish protection against WT *L. monocytogenes* or virus challenge compared to that of KBMA vaccines with native *prfA*. In a dose-response experiment, the superior potency of KBMA PrfA*(G155S) for inducing B8R responses could be seen compared to levels for mice immunized with KBMA harboring WT *prfA* (Fig. 5A).

A relevant measure of vaccine-induced T-cell potency is protective immunity against challenge with a live pathogen. We evaluated T-cell potency in mice immunized with KBMA PrfA*(G155S) or WT *prfA* by challenge with WT *L. monocy-*

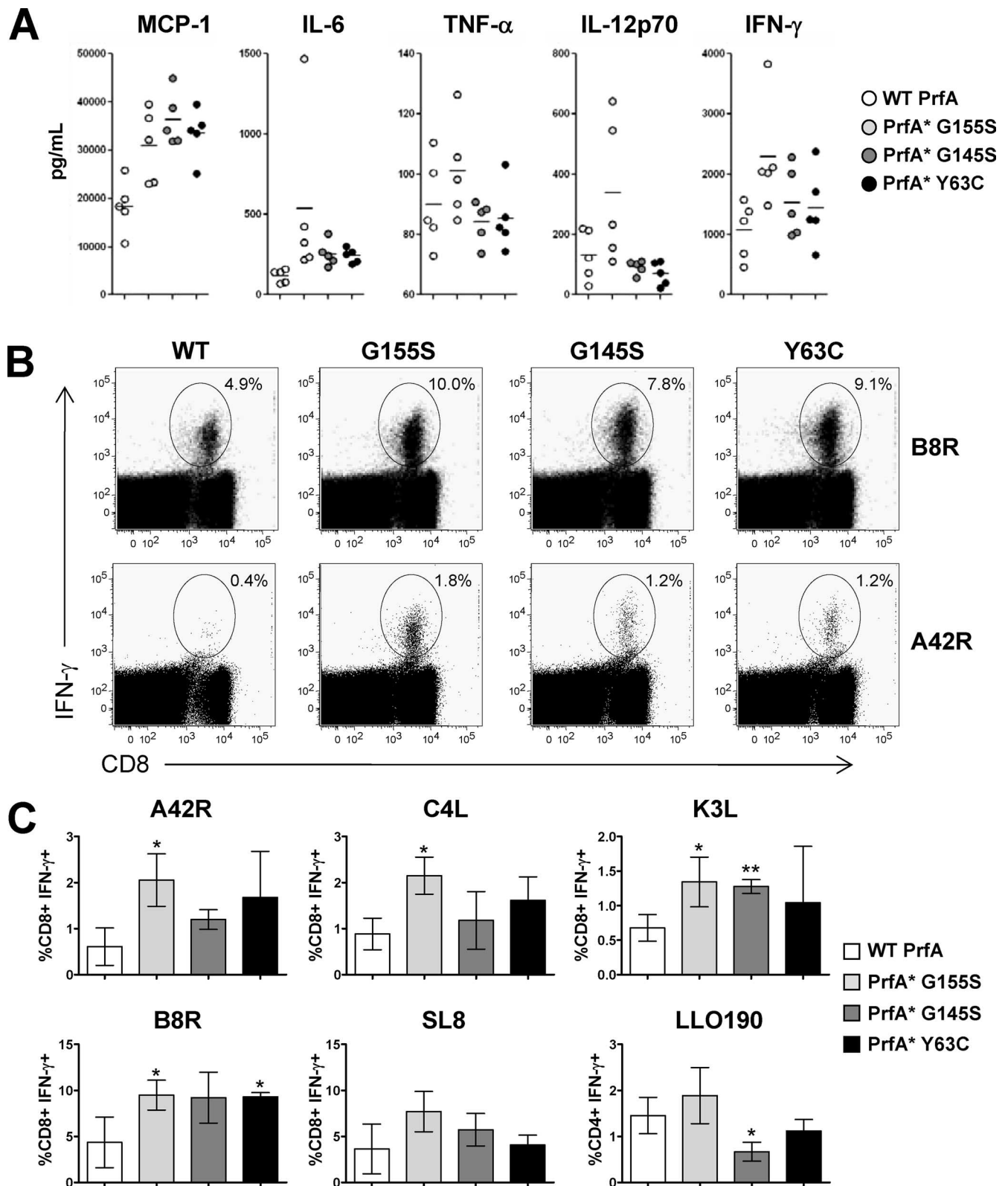


FIG. 3. PrfA* enhances the immunogenicity of KBMA *L. monocytogenes* vaccines. (A) Serum cytokine/chemokine levels determined 8 h following a single i.v. administration of 1×10^8 particles of KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA*, PrfA*(G155S), PrfA*(G145S), and PrfA*(Y63C) strains. Cytokine/chemokine levels were determined by cytometric bead analysis. Each symbol represents a single animal. The levels of monocyte chemoattractant protein 1 (MCP-1) were significantly increased compared to the WT levels for PrfA*(G155S) ($P < 0.05$), PrfA*(G145S), and PrfA*(Y63C) ($P < 0.005$). PrfA*(G155S) increased the level of IFN- γ ($P < 0.05$). The levels of IL-6 were increased in PrfA*(G145S) ($P < 0.05$) and PrfA*(Y63C) ($P < 0.005$). (B, C) KBMA *L. monocytogenes* PrfA* strains induced Ag-specific immunity of a higher magnitude. C57BL/6 mice were immunized i.v. with 1×10^8 particles of KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA*, PrfA*(G155S), PrfA*(G145S), and PrfA*(Y63C) strains. Ag-specific T-cell responses were determined by intracellular cytokine staining at the peak of the response 7 days following vaccination. (B) Dot blots from a representative animal from each group are shown. (C) The means \pm standard deviations are shown for each group of five animals. *, $P < 0.05$; **, $P < 0.005$ for comparison to WT *prfA*.

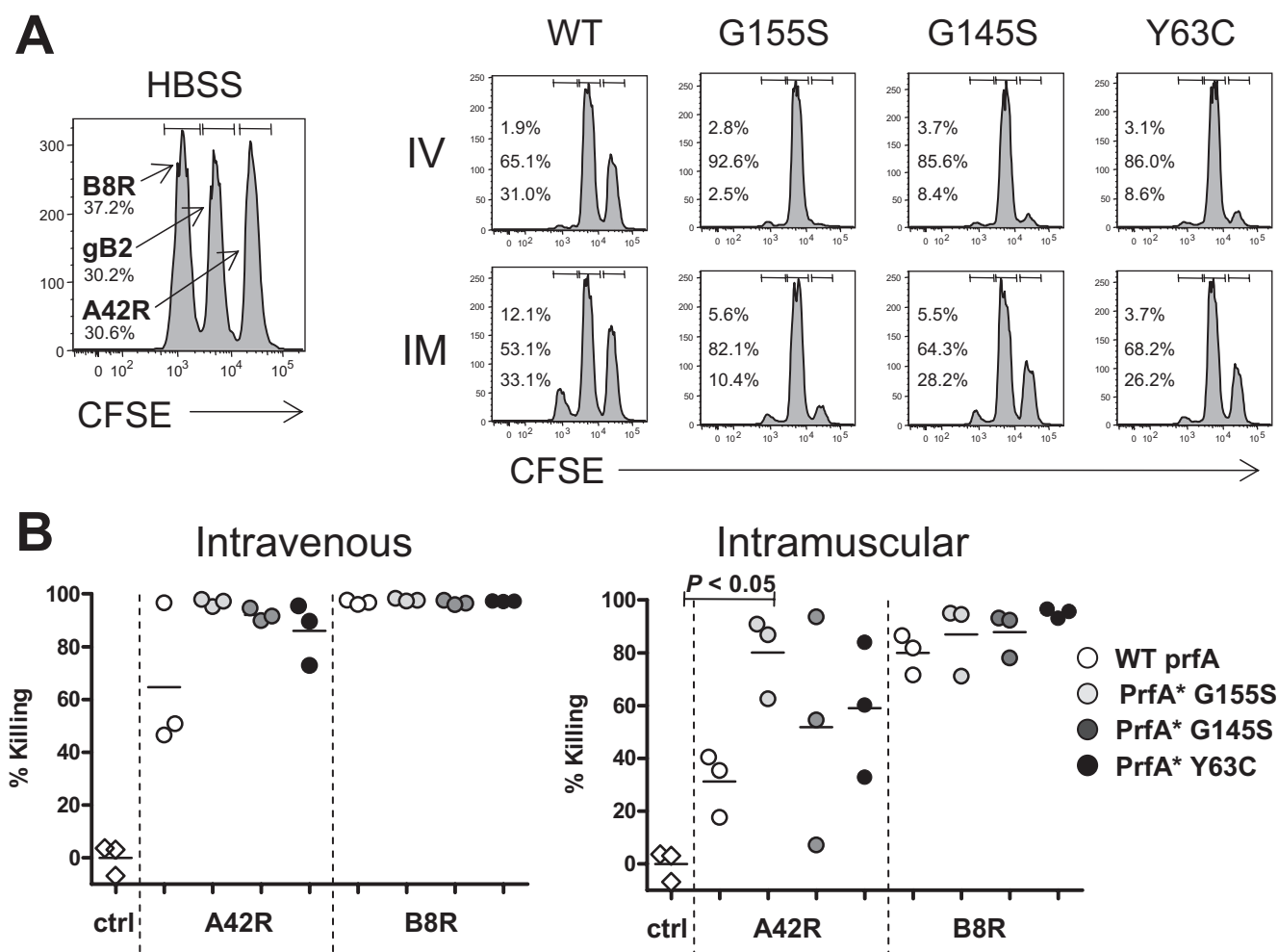


FIG. 4. Improved potency of T-cell responses elicited by KBMA *L. monocytogenes* PrfA* strains. (A, B) C57BL/6 mice were immunized i.v. or i.m. (as indicated in the figure) twice, 2 weeks apart, with HBSS (left) or with 1×10^8 particles of KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT prfA, PrfA*(G155S), PrfA*(G145S), and PrfA*(Y63C) strains. In vivo cytolytic activity was determined 7 days later by challenging mice with gB2 (control; middle peak), A24R-loaded targets (right peak), or B8R-loaded targets (left peak). (A) A histogram for a representative animal is shown for each group. (B) In vivo cytolytic activity specific to A42R and B8R is shown for mice vaccinated i.v. or i.m. Each symbol represents an individual animal. The A42R response in i.m. vaccinated mice was significantly higher for the PrfA*(G155S) strain than for the WT ($P < 0.05$).

*to*genes or vaccinia virus. Strains of *L. monocytogenes* that fail to escape from the phagolysosome fail to induce protective immunity, although Ag-specific T-cell responses that can be expanded upon secondary challenge are elicited (4, 18). We previously described that KBMA *L. monocytogenes*-based vaccination results in transient protection against a lethal WT *L. monocytogenes* challenge. The induced T-cell response wanes over time, reminiscent of a T-cell response induced in the absence of CD4⁺ T-cell help (42; K. S. Bahjat, unpublished data). Immunization of mice with the KBMA *L. monocytogenes* $\Delta actA \Delta inlB \Delta uvrAB$ strain resulted in a 2-log protection at 14 days. To evaluate the potency of the KBMA PrfA*(G155S) vaccine-induced adaptive response, mice were immunized once with 1×10^8 particles and challenged with a $2 \times LD_{50}$ with WT *L. monocytogenes* 14 days later. CFU counts in spleen and liver were determined (data not shown) 3 days later. As shown in Fig. 5B, protection from WT *L. monocytogenes* was improved by 3 logs in mice immunized with KBMA PrfA*(G155S) com-

pared to levels for mice immunized with the KBMA strain with WT prfA.

We then determined whether the enhanced immunologic potency of KBMA PrfA*(G155S) also extended to vaccinia virus challenge. C57BL/6 mice were immunized twice by an i.m. route 2 weeks apart with KBMA PrfA*(G155S) or WT prfA vaccines encoding the Quadvac Ag. To evaluate protective memory immunity, mice were challenged with 1×10^7 PFU of vaccinia virus 30 days following the last immunization and viral titers in the ovaries of the mice were determined 5 days later. Consistent with the improved magnitude of the induced T-cell response to the various vaccinia virus epitopes, we observed a statistically significantly improved protection by 2 logs against viral challenge in mice that received the PrfA*(G155S) mutant on the background of the KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ strain (Fig. 5C).

These results demonstrate that PrfA*(G155S) confers increased immunologic potency to live-attenuated and KBMA *L.*

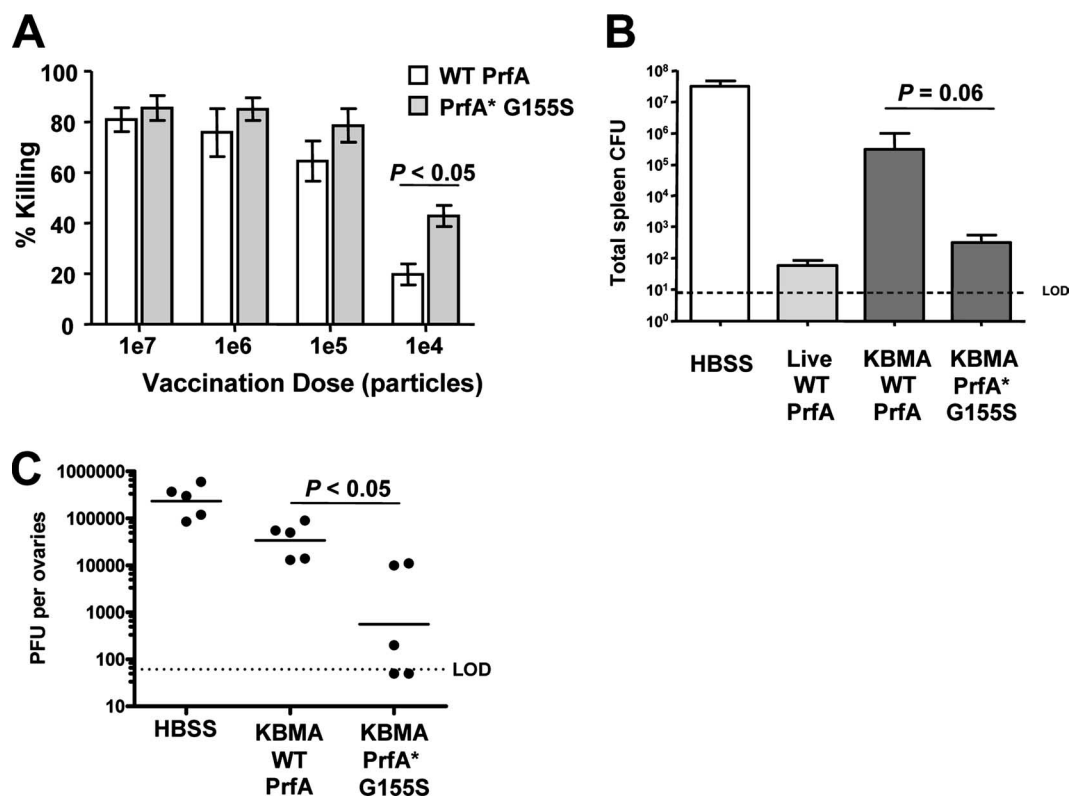


FIG. 5. The *PrfA**(G155S) vaccine strain induces protective immunity. (A) In vivo cytolytic activity specific to B8R is shown following two vaccinations 2 weeks apart at various doses of the KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA* or *PrfA**(G155S) strain. The means \pm standard deviations are shown for groups with five animals each. The difference between WT *prfA* and *PrfA**(G155S) vaccine strains is statistically significant at the 1×10^4 -CFU vaccination dose. (B) Protective immunity to a $2 \times LD_{50}$ challenge with WT *L. monocytogenes* is shown. BALB/c mice were immunized i.v. once with 1×10^8 particles of the KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA* or *PrfA**(G155S) strain. HBSS served as a control. Spleens were harvested 3 days after challenge and plated for CFU. (C) Viral titers in ovaries following an intraperitoneal challenge with 1×10^7 PFU of vaccinia virus. C57BL/6 mice were vaccinated twice i.v. with 1×10^8 particles of the KBMA *L. monocytogenes* $\Delta actA \Delta inlB$ WT *prfA* or *PrfA**(G155S) strain. Viral titers were determined 5 days after vaccinia virus challenge. Each symbol represents an individual animal. The log difference in protection between WT *prfA* and *PrfA**(G155S) is statistically significant.

monocytogenes vaccines, notably providing the ability of KBMA vaccines to elicit protective immunity in rigorous infectious-disease challenge models following a conventional immunization route.

DISCUSSION

Recombinant vectors derived from viruses or bacteria represent an attractive but as-yet-unproven approach to stimulating functional immune responses in human prophylactic or therapeutic disease settings. Despite the challenges that face the multiple technologies under development, efforts in the field of recombinant vector platforms continue to be substantial due to their potential to address significant unmet medical needs. Recent high-profile failures of vaccine clinical trials, such as reports detailing the failure of an adenovirus serotype 5-based preventative vaccine in advanced testing to protect against human immunodeficiency virus infection (this vaccine may have even augmented the infection), underline the acute need for effective new vaccine platforms (12, 13, 33). Vaccine platforms based on live-attenuated *L. monocytogenes* are being developed and evaluated clinically due to an inherent property of stimulating potent innate immunity and acquired cellular

immunity (identifiers NCT00327652 and NCT00585845 [http://www.clinicaltrials.gov/]). In this investigation, we show that activating the *PrfA* regulon prior to vaccinating mice enhanced the levels of innate and cellular immunity induced by live-attenuated and KBMA *L. monocytogenes* vaccines, which correlated with improved protection against challenge with the cognate WT bacterial pathogen or vaccinia virus. These results form the basis of a rationale for including the *prfA*(G155S) allele in future *L. monocytogenes*-based vaccines advanced to the clinical setting.

The immune potencies for both live-attenuated and KBMA vaccines were enhanced by activation of the *PrfA* regulon prior to immunization. *PrfA** significantly enhanced the immune potency of KBMA vaccines. We have shown previously that, although killed, KBMA vaccines still escape the phagolysosome, a necessary step toward inducing IFN- β and other activating signals in APCs required to elicit protective cellular immunity against challenge with WT *L. monocytogenes* (4, 8, 27). However, KBMA *L. monocytogenes* vaccines can elicit protective immunity after a single immunization only when administered in combination with surrogate help provided by anti-CD40 antibody or when a homologous prime and boost immunization regimen is used (4). These results demonstrate a

reduced immune potency for KBMA vaccines compared to that for live-attenuated *L. monocytogenes* $\Delta actA \Delta inlB$ vaccine strains, which, like WT *L. monocytogenes*, can elicit protective immunity after a single immunization. Live *L. monocytogenes* strains expanded 100-fold over 7 h in the cytoplasm of infected macrophages in vitro (Fig. 1E), resulting in full activation of PrfA and induced expression of *prfA*-dependent genes, including encoded Ags which were driven from the *actA* promoter. In contrast, KBMA vaccines are unable to propagate in cells of the immunized host, and under conditions of a nonreplicating vector, induction of the PrfA regulon and expression of an encoded Ag prior to vaccination significantly enhanced immune potency.

In a recently published study, the immunogenicity of WT *L. monocytogenes* strain 10403 was compared with that of a different WT *L. monocytogenes* strain, 43251, which contains an unknown activating *prfA* mutation (39). While the latter *L. monocytogenes* strain elicited enhanced LLO- and p60-specific immunity and increased protection against WT bacterial challenge, because this study utilized different WT *L. monocytogenes* strains, it is difficult to draw conclusions regarding underlying mechanisms and possible application to recombinant attenuated vaccine platforms that are appropriate for testing with humans. Furthermore, the impact of constitutive PrfA activation on the immunogenicity of expressed heterologous Ags in recombinant *L. monocytogenes* vaccine strains was not evaluated in this study.

The enhanced immune potency of KBMA PrfA*-based vaccines could be due to several independent mechanisms. Contributing factors may include increased efficiency of escape from the phagolysosome and increased Ag expression and secretion in the cytosol, ultimately resulting in a higher density of epitopes displayed on MHC class I molecules and more efficient priming of CD8⁺ T cells. While overexpression of PrfA-dependent virulence genes can increase cytotoxicity, resulting in decreased virulence of WT strains and decreased vaccine potency (9, 15, 43), this mechanism does not appear to have affected the relative immunogenicities of the vaccine strains used in this study, as shown by equivalent intracellular growth in J774 cells (Fig. 1E). Other possibilities may include enhanced migration of DCs to the lymph nodes of animals immunized with PrfA* vaccines, due to an improved proinflammatory cytokine milieu at the site of infection or increased InlA-mediated disruption of E-cadherin DC-DC adhesions (20). Although binding of InlA to mouse E-cadherin is diminished compared to binding to its human homolog (22, 46), increased levels of InlA from PrfA* strains may still enhance this process. On the other hand, it seems unlikely that the enhanced immune potency of KBMA PrfA* vaccines was due to either an increased host range or an enhanced infection of target cells. Notably, while important for oral infection, for the i.m. or i.v. routes used in this study, InlA does not play the same role in mediating infection of nonphagocytic cells. Furthermore, InlB-mediated infection of hepatocytes via the hepatocyte growth factor receptor is not relevant, since this virulence determinant was deleted from the vaccine strains used in this study. Supporting this notion are the combined observations that infections of cultured macrophages or DCs were indistinguishable between all of the vaccine strains tested (Fig. 1C and D) and that the virulence of *L. monocytogenes* $\Delta actA$

$\Delta inlB$ PrfA* strains was increased only approximately twofold over that of the *L. monocytogenes* $\Delta actA \Delta inlB$ parent strain, which is attenuated by more than 3 logs compared to WT *L. monocytogenes* (9) (Table 1). We are currently investigating whether any of these mechanisms contribute to the increased potency of KBMA PrfA* vaccines.

While the results presented here demonstrate the importance of activation of the PrfA regulon for increasing the potency of *L. monocytogenes* vaccines, increased Ag expression in cultured APCs infected with PrfA* vaccine strains was not observed. These results were somewhat surprising since previous studies showed increased actin polymerization in PtK2 cells infected with PrfA* mutants on a WT *L. monocytogenes* background, potentially due to increased or earlier expression of ActA, although ActA protein levels were not directly measured (38). Nevertheless, *prfA*(G155S), *prfA*(G145S), and *prfA*(Y63C) mutations all conferred high (and equivalent) levels of overexpression of PrfA-dependent Ags in vaccine strains grown in broth culture, but only PrfA*(G155S) vaccine strains had significantly increased immunologic potency. Interestingly, the live-attenuated PrfA*(G155S) and PrfA(G145S) [but not the PrfA*(Y63C)] vaccine strains elicited higher-magnitude T-cell responses to p60, a non-PrfA-dependent *L. monocytogenes* Ag, indicating that improved immunogenicity may not only be a consequence of improved Ag expression levels (data not shown). Thus, it is not surprising that enhanced PrfA-dependent expression did not necessarily correlate with optimal immunogenicity, since host-pathogen interactions are by definition a complex multifactorial process. Temporal regulation of PrfA-dependent genes provides expression of particular bacterial proteins in appropriate cellular compartments to facilitate pathogenesis. For example, ActA expression is induced 200-fold in the cytoplasm to promote host cell actin polymerization and cell-to-cell spread (32, 37). The Ags in this study were expressed as an N-terminal fusion with the first 100 amino acids of ActA and driven from a native *actA* promoter. In the case of KBMA vaccines, *prfA*(G155S) provided the appropriate level of PrfA-dependent induction to augment potency but afforded a sufficient balance of metabolic economy for the photochemically inactivated bacterium. In a recent study utilizing *prfA*(L104F) to characterize the PrfA-dependent *L. monocytogenes* secretome (31), several proteins whose expression was not known previously to be related to activated PrfA were identified. These data provide evidence for the multiple bacterial proteins involved in the pathogenesis of WT *L. monocytogenes* and illustrate that PrfA* mutants may have a complex impact on the potency of *L. monocytogenes* vaccines.

The overwhelming majority of preclinical studies with *L. monocytogenes* vaccines have utilized either i.v. or intraperitoneal administration. There have been few reports examining i.m. and subcutaneous immunization routes (9, 14). However, oral immunization has been explored in studies with mice and nonhuman primates and a single study with humans (2, 7, 26, 30). The three clinical trials with *L. monocytogenes* conducted to date or ongoing have utilized i.v. administration. While KBMA *L. monocytogenes* vaccines may have an improved risk-to-benefit profile compared to live-attenuated vaccines, a traditional immunization route of administration may be necessary for broad adoption and/or approval, particularly in prophylactic settings. Here, we show that with a prime-boost

immunization regimen following i.m. immunization, KBMA PrfA*(G155S) vaccines elicited functional cellular immunity that was comparable to that elicited by live-attenuated *L. monocytogenes* vaccines.

By evaluating the immune potency of a panel of isogenic live-attenuated and KBMA *L. monocytogenes* vaccine strains varying only in *prfA*, we have shown that constitutive induction of the PrfA regulon prior to immunization enhances the ability of live-attenuated KBMA vaccines to elicit functional cellular immunity, using a conventional immunization route. We are presently evaluating this platform with various infectious-disease applications, advancing toward eventual testing of KBMA PrfA*(G155S) vaccines in the clinical setting.

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